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Ionising radiation induces changes associated with epithelial-mesenchymal transdifferentiation and increased cell motility of A549 lung epithelial cells

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ABSTRACT

Radiotherapy remains a major therapeutic option for patients with advanced lung cancer. Nevertheless, the effects of irradiation on malignant biological behaviours (e.g. migration and transformation of cancer cells) have yet to be clarified. We conducted an *in vitro* study to investigate the radiation-induced alterations including morphology, adhesion, and cell motility of A549 human lung cancer cells. These changes, which are associated with epithelial-mesenchymal transdifferentiation (EMT), seem to be linked to radiation-induced fibrosis, which represents one of the most common long-term adverse effects of curative radiotherapy. In addition, loss of intercellular adhesion and increased cell motility may be involved in post-radiotherapy-associated metastasis. We showed that stress fibres and focal adhesions are increased and that cell–cell junctions are decreased in response to ionising radiation. Radiation also significantly increased cell motility. The p38-specific inhibitor, SB203580, reduced the radiation-promoted migration of A549 cells, whereas SP600125, a JNK MAPK-specific inhibitor, inhibited both inherent and radiation-mediated cell motility. Consistent with this observation, radiation up-regulated the phosphorylation of p38 MAPK. Current approaches to cancer treatment involving more intensive radiotherapy regimens have been suggested to be associated with a higher incidence of local or distant metastasis. Therefore, a subset of patients may benefit from a combination of radiotherapy with inhibitors of EMT or cell migration.

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1. Introduction

Radiation therapy is a common conventional treatment modality for various human solid tumours. However, the therapeutic efficacy of radiotherapy alone for treating locally or regionally advanced cancer is often limited by tumour radioresistance, systemic tumour progression and local or distance metastases.[1–3] In particular, numerous researchers have observed that treatment of the primary tumour with either surgery or radiotherapy can have unpredictable effects on metastases. The metastatic nature of lung cancer has been responsible for the poor survival statistics and emphasises the need for the effective suppression of metastases. The process of cell migration and invasion during metastasis is frequently associated with the loss of epithelial markers and the acquisition of mesenchymal markers as well as increased migration and motility, cellular changes collectively known as epithelial-mesenchymal transdifferentiation (EMT).[4]

Cell–matrix and cell–cell adhesion are distinguishing features of multicellular organisms and play crucial roles in the maintenance of epithelial integrity.[5–7] EMTs occur during critical phases of embryonic development in many animal species. In fact, the phenotypic changes of increased motility and invasiveness of cancer cells are reminiscent of the EMTs that occur during embryonic development. EMT is considered to be a highly regulated process that consists of four key steps: loss of epithelial cell adhesion; *de novo* expression of α-smooth muscle actin (α-SMA) and reorganisation of actin; disruption of tubular basement membranes; and enhanced cell migration and invasion.[8–10]

In many cells in culture, integrins are clustered at sites of adhesion to the underlying extracellular matrix (ECM) in regions known as focal adhesions or focal contacts (for review, see Ref. [11]). The extracellular face of focal adhesions mediates attachment to matrix components adsorbed onto the plastic or glass culture surface, whereas the cytoplasmic face provides a site of attachment for bundles of actin filaments (stress fibres). Engagement of cell surface integrins is associated with rapid tyrosine phosphorylation of several intracellular proteins, including focal adhesion kinase (FAK), paxillin, tensin, and $p130^{CAS}$. FAK and paxillin are two focal adhesion-associated proteins that function in transmitting signals downstream of integrins.[12] These signals control important biological events, including cell migration, proliferation, and survival. Many reports have implicated FAK and paxillin as positive regulators of cell motility,[13,14] although there are contradictory reports that they act as negative regulators.[12]

The role of different signalling pathways in inducing reversible or irreversible EMT has been studied in several cell culture models. These investigations have shown that many signalling proteins are involved in EMT.[4] Recent studies have demonstrated that mitogen-activated protein kinases (MAPKs), including Jun N-terminus kinase (JNK), p38, and extracellular regulated kinase (ERK), play crucial roles in cell migration.[15] Of these, JNK is generally thought to be involved in inflammation, proliferation, and apoptosis.[16,17] It has also been shown to be essential for cell migration and required for rapid movement, suggesting it as a possible critical signal of EMT. However, it is also reported that inhibition of JNK with curcumin[18] or by expression of a dominant-negative JNK mutant[19] does not affect EMT. Therefore, the function of JNK in EMT is controversial. Furthermore, because few studies have examined the role of p38 MAPK in EMT, its function in these cellular changes is also unclear.

Radiation fibrosis is a frequent sequel of therapeutic or accidental radiation overexposure in cancerous or normal human tissues.[20] One of the fundamental problems remaining unsolved in this regard is the origin of the chronic activation of myofibroblasts within fibrotic tissues. In this context, conversion of epithelial cells to α-SMA-expressing myofibroblasts is a key feature of radiation fibrosis. It has been postulated that ionising radiation (IR) results in the continuous production of tissue repair activating factors including various cytokines and growth factors. Among these, transforming growth factor-β1 (TGF-β1) is considered a master switch for EMT and the resulting fibrotic program.[21] TGF-β1 plays a central role in fibrosis, contributing to the influx and activation of inflammatory cells, EMT of cells, and the influx of fibroblasts and their subsequent regulation of ECM.[9] Although radiation fibrosis has been reported for many years in histopathological studies, the mechanisms of its initiation and chronic extension still remain to be determined.[22]

Here we demonstrate that IR induces a series of EMT-associated changes in A549 lung cancer cells and that p38 MAPK signalling seems to play a critical role in radiation-induced cell motility. EMT-associated cell migration play an important role in primary tumour progression.[23] Therefore, this pathway could be a target for therapeutic intervention of invasion, metastasis as well as fibrosis associated with radiation therapy.

2. Materials and methods

2.1. Cell culture conditions and irradiation of cells

A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). 1×10^6 cells in 60 mm culture dish were exposed to acute doses of 2, 4, 6, 8, or 12 Gy external beam photon irradiation using a Cesium irradiator at a dose rate of 2.32 Gy/min (Cs-137 Model 68; JL Shepherd and Associates, Glendale, CA) delivering 7.3 Gy/min at room temperature. For some conditions, cells were pretreated with inhibitors for 1 h before irradiation. Cell morphology was observed under the phase contrast inverted microscope (Leica, DMIL). Porcine TGF-β1 was purchase from R&D system (Minneapolis, MN, USA).

2.2. Transfection and luciferase assay

TGF-β responsiveness of irradiated A549 cells was also assessed with a reporter assay using p3TP-lux construct, which contains three tandem transforming growth factor β-Smad

signalling responsive elements.[24] A549 cells (3×10^4) were transfected with the p3TP-Lux reporter plasmid and the CMV-βGal plasmid in 10:1 ratio using LipofectAMINE™ according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After overnight transfection, the cells were allowed to recover for 10 h in DMEM containing 5% FBS and were treated with or without irradiation (6 or 12 Gy) and maintained for 24 h. Luciferase activity was measured using the luciferase assay reagent following the manufacturer's protocol (Promega, Madison, WI USA). Bioluminescence was measured using a Turner Designs luminometer (TD-20/20).

2.3. Cell adhesion assay

Cell adhesion assay was performed as described previously.[25] In brief, A549 cells were incubated in DMEM supplemented with 1% FBS for 12 h and harvested by bathing in 1 mM EDTA for 10 min. The cells were counted with haemacytometer and then treated with radiation (0, 2, 4, 6, or 8 Gy) in suspension prior to plating 2×10^4 cells/well on 96 well plates in DMEM for 30 min. Non-adherent cells were removed by washing twice with phosphate-buffered saline, and the remaining cells were quantitated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasolium bromide (MTT) method. For MTT assay, 50 μl of 5 mg/ml MTT (Sigma-Aldrich, St Louis, MO USA) in growth medium was added to each well. After incubation for 2 h at 37 °C with MTT, cell medium was removed. The precipitated formazan, a product of MTT tetrazolium ring by the action of mitochondrial dehydrogenases, was solubilised with dimethyl sulfoxide and quantified spectrophotometrically at 540 nm. Viability of non-adherent cells was verified by trypan blue exclusion.

2.4. Immunofluorescence microscopy

A549 cells (10^5cells/well) were grown in DMEM containing 5% FBS on glass coverslips (22×22 mm) for 24 h before irradiation. Cells were then subjected to irradiation for single dose of 6 or 12 Gy. After 24 h, cells were fixed in 4% paraformaldehyde, permeabilised in PBS containing 0.1% Triton X-100. Cells were blocked with 3% skim milk in PBS for 1 h at RT, incubated for 2 h with primary antibodies diluted in 1% skim milk/PBS (1/300 for paxillin, 1/500 for p-Tyr, 1/200 for anti-phosphorylated H2AX (γ-H2AX) or 1/250 for E-cadherin). After extensive washing, fluorescent secondary antibodies (1/500) in PBS were added for 60 min at RT. The coverslips were washed again and mounted on slides using Fluorescence Mounting Medium (DAKO Diagnostics, Mississauga, ON USA). Actin organisation was visualised by staining with FITC-conjugated phalloidin (Molecular Probes, Eugene, OR USA, Cat # A12379). Anti-phospho-tyrosine antibody (4G10) (hybridoma ascites) and anti-paxillin (Transduction laboratories, Lexington, USA, Cat # 610619) were used to detect immunofluorescence of focal adhesion, anti-E-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat # SC-8426) was used to examine cell–cell contact and γ-H2AX antibody (Transduction laboratories, Lexington, USA, Cat # 07-164) was used to detect DNA double strand break. Slides were analysed by confocal laser scanning microscope (Zeiss LSM 510 META). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

2.5. Motility and migration assays

The scratch motility assay was used to measure two-dimensional movement. A549 cells were grown to confluence in 6-well plates. A scratch was then made on the monolayer using a sterile 200 μl-pipette tip. The monolayer was rinsed three times with and placed in DMEM with 1% FCS followed by irradiation. For some conditions, cells were treated with inhibitors (PD98059, SB203580 or SP600125) 30 min before irradiation. Phase contrast images were captured after 24 or 48 h incubation with inhibitors and a digital image of the scar was taken at a magnification of ×20 (Leica, DMIL).

To measure three-dimensional movement, the Transwell migration assay was used. A549 cells (1×10^5/well) were plated in DMEM/1% FBS in the upper chamber of 5 μm pore (24-well) transwells (Costar, High Wycombe UK) and allowed to adhere for 2 h and irradiated. For some conditions, cells were treated with inhibitors (PD98059, SB203580 or SP600125) 30 min before irradiation. The cells were returned to the incubator for 24 h, rinsed with PBS, fixed in 100% methanol and cells remaining at the top of the polycarbonate membrane were removed. The cells that had migrated through pores to the lower surface were stained with ethanol-based crystal violet solution. Membranes were mounted on microslides and cells were counted.

2.6. RT-PCR

Total cellular RNA was extracted with Trizol™ (Gibco, Grand Island, NY USA) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed for 1 h at 37 °C in a reaction mixture containing 5 U RNase inhibitor (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTP (Böehringer Mannheim, Indianapolis, IN, USA), oligo dT primer, 1× RT buffer and 5 U Omniscript™ reverse transcriptase (Qiagen, Santa Clarita, CA, USA) in a 50 μl reaction. The mixture was placed at 37 °C for 60 min and then rapidly cooled on ice. cDNA (1 μl) was PCR amplified. For PCR reaction of alpha smooth muscle actin (α-SMA), vimentin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the following conditions were used: one cycle at 95 °C for 2 min followed by 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s, for 27 cycles, and one cycle at 72 °C for 7 min. PCR primers are as below: α-SMA (forward, F: 5′-ccgggagaaaatgactcaaa-3′, reverse, R: 5′-gaaggaatagccacgctcag-3′), vimentin (F: 5′-ggctcagattcaggaacagc-3′, R: 5′-gcttcaacggcaaagttctc-3′) and GAPDH (F: 5′-tcattgacctcaactacatggt-3′, R: 5′-ctaagcagttggtggtgcag-3′). Analysis of the resulting PCR products on 1% agarose gels showed single-band amplification products with expected sizes.

2.7. TGF-β1 assay

Measurement of TGF-β1 in the culture medium was achieved by enzyme-linked immunosorbent assay (ELISA) using multi-species TGF-β1 ELISA kit according to manufacturer specifications (BioSource International, Inc. Camarillo, CA, USA). A 200 μL aliquot of medium was used for each assay and the relative

values were compared before and after irradiation. Medium alone without cells was incubated under the same conditions and used as a blank control for the TGF-β1.

2.8. Immunoblotting

Whole cell protein lysates of A549 cells were prepared in lysis buffer (10 mM Tris–HCl pH 8.0, 140 mM NaCl, 1% Triton, 0.5% SDS and protease inhibitors) and cleared from cellular debris by centrifugation. The supernatants were aliquoted and stored at −80 °C for further use. Samples were assayed for protein concentration using Bradford (BioRad, Richmond, CA USA) assay. Protein samples (20–100 μg for each) were separated by 10% SDS-PAGE and transferred to Hybond^{TM}-ECL^{TM} nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ USA). The membrane was blocked with 5% bovine serum albumin (BSA) in TBST solution (10 mM Tris-HCl [pH 8.0] containing 150 mM NaCl and 1% Tween 20). The blots were incubated with anti-125^{FAK} mAb (Santa Cruz Biotechnology, Cat # SC-557), anti-phosphotyrosine397-125^{FAK} (BioSource International, Inc, Cat # 44-624G), anti-paxillin mAb (Transduction Laboratories, Lexington USA), polyclonal antiphospho-p38 MAPK (Cell Signalling Technology, Beverly, MA USA, Cat # 9211), antiphospho-ERK (Santa Cruz Biotechnology, Cat # SC-7383), antiphospho-JNK (Cell Signalling Technology, Cat # 9251), anti-p38 MAPK (Cell Signalling Technology, Cat # 9212), anti-ERK (Santa Cruz Biotechnology, Cat # SC-154), or anti-JNK (Santa Cruz Biotechnology, Cat # SC-474) in blocking solution overnight at 4 °C. After washing with TBST, horse radish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences) (1:10,000 dilution in TBST) were applied and the blots developed by the Enhanced Chemiluminescence (ECL) detection system (Amersham Biosciences).

2.9. Statistical analysis

The data are expressed as the mean ±SEM and analysed for statistical significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparison. A p value <0.05 was considered significant.

3. Results

3.1. IR causes changes in the morphology and adhesion of A549 cells

Subjecting A549 human epithelial lung adenocarcinoma cells to IR (4, 6, or 8 Gy) before 24 h incubation caused a dramatic and dose-dependent change in cell morphology from cuboidal to an elongated spindle-like shape (Fig. 1a). These changes were sustained for at least 72 h (data not shown). Nonirradiated control cells were found to form focuses of tightly clustered cells, with virtually every cell in the field incorporated into the clusters (Fig. 1a). In contrast, tightly clustered cells were not observed in cells treated with IR. These changes were likely accompanied by the rearrangement of cell–cell or cell-ECM focal contacts. Therefore, we examined whether IR affects cell adhesion. We found that subjecting A549 cells to single dose of 2, 4, 6, or 8 Gy of IR for 24 h caused a significant and dose-dependent increase in cell adhesion (Fig. 1b).

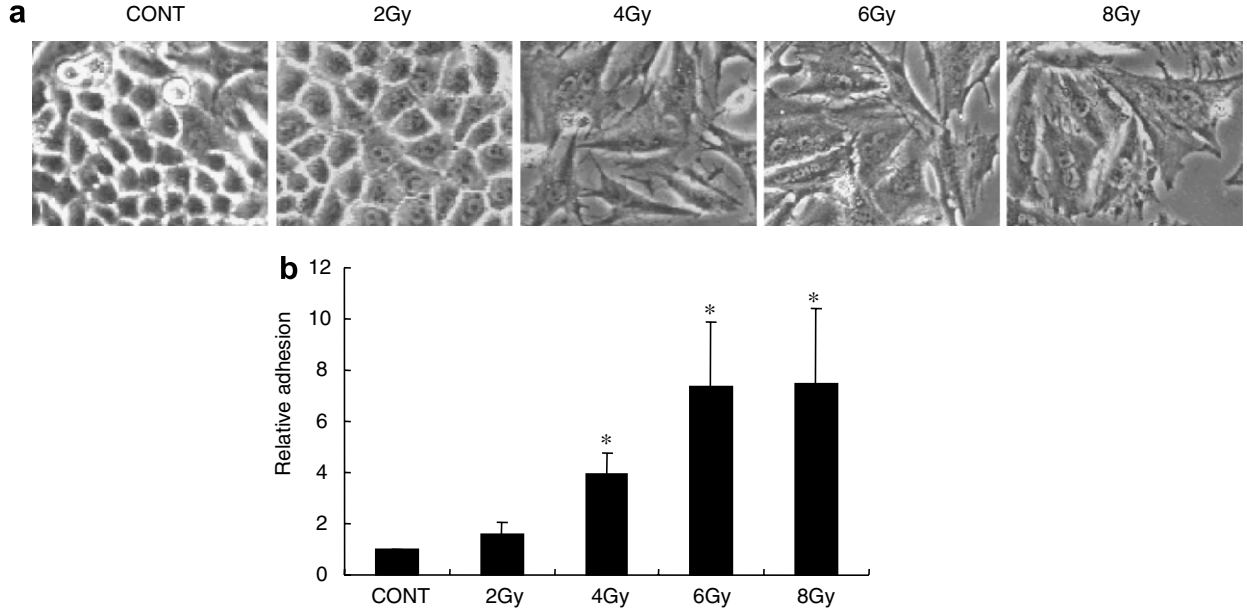

Fig. 1 – IR induces changes in the morphology and adhesion of A549 lung cancer cells. A549 cells (2×10^6) were subjected to a single dose of IR (0, 2, 4, 6, or 8 Gy) using a gamma cell irradiator. (a) After 24 h, cell morphology was observed by phase contrast microscopy. (b) Quantification of adhesion assays. Irradiated or untreated A549 cells were plated on 96-well plates for 30 min, rinsed, stained with MTT, and quantified. Measurements were made in triplicate, and data are representative of three separate experiments. The *error bars* indicate the standard deviation (SD) $^*p < 0.05$ versus nonirradiated control cells.

3.2. *IR effects on focal adhesion complexes, the actin cytoskeleton, and cell–cell interaction*

The phenotypic transition of A549 epithelial cells to an elongated spindle-like shape with extensive filopodia and increased cell adhesion seems to be potentially linked with rearrangement of cytoskeleton or focal contact. To determine whether radiation induces changes in actin stress fibre formation, we fluorescent stained the cells for polymerised-actin by using fluorescein isothiocyanate-conjugated phalloidin. In addition, assembly of focal contacts was visualised with anti-phosphotyrosine and anti-paxillin antibodies,[26–28] and cell–cell interaction was examined using an anti-E-cadherin antibody. As shown in Fig. 2, treatment with IR caused an increased actin stress fibre immunostaining but a decrease or reorganised E-cadherin immunoreactivity in 24 h. The amount of E-cadherin measured by western blotting and RT-PCR was not significantly changed by IR (data not shown), suggesting that IR likely redistributed subcellular localisation of E-cadherin rather than down regulation of protein level. Staining of phosphotyrosine or paxillin displayed more intensely at focal contact. Intense phosphotyrosine staining is typically associated with focal contacts at cell peripheries or nascent contacts in which FAK is activated.[28] Such changes in actin cytoskeleton, focal contact or E-cadherin after IR are reminiscent of the EMT.[29] IR-treated cells were analysed by immunofluorescence for phosphorylated form of the histone variant H2AX(γ-H2AX) protein which is known to accumulate at sites of breaks produced by IR.[30] Clear γ-H2AX foci were observed in IR-treated A549 cells at 24 h.

3.3. *Radiation increases cell motility in A549 lung cancer cells*

Another important characteristic of EMT involves the acquisition of motile behaviour and the ability to migrate. Therefore, we investigated the effect of IR on the motility of A549 cells. The scratch motility assay was used to measure two-dimensional movement (Fig. 3a). A549 cells were grown to confluence and a scratch was made using a sterile 200-μl pipette tip. The monolayer was rinsed three times followed by irradiation (6 or 12 Gy). Phase contrast images captured after 24 or 48 h incubation showed IR increased wound healing activity, indicating that IR upregulated cell motility. In order to minimise cell proliferation, cells were incubated in 1% FBS containing medium. To measure three-dimensional movement, the Transwell migration assay was used. A549 cells were plated in the upper chamber of 5 μm pore transwells and allowed to adhere for 2 h and irradiated. Inhibitors (PD98059, SB203580 or SP600125) were treated 30 min before irradiation. The cells incubated for 24 h and cells migrated to the bottom of the transwell filter were counted. The results of these assays showed that IR significantly increases the motility of the cells and their ability to migrate (Fig. 3a and b).

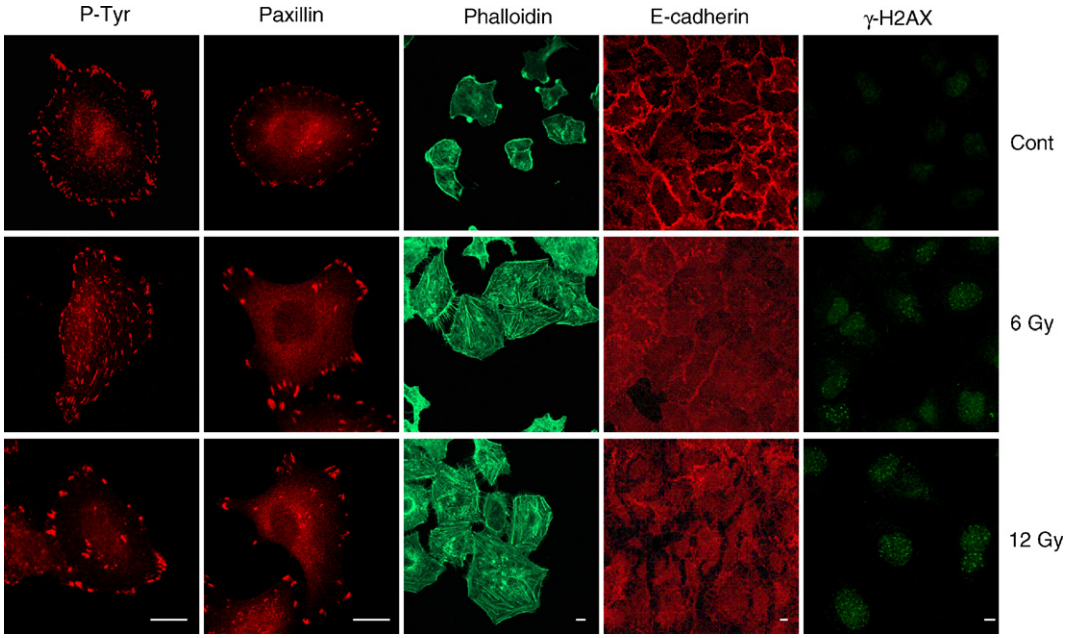

Fig. 2 – IR causes a significant change in phosphotyrosine, paxillin, F-actin, and E-cadherin immunoreactivity and localisation in A549 cells. A549 cells were subjected to a single dose of IR (6 or 12 Gy) using a gamma cell irradiator. After 24 h, focal adhesion complexes were stained with an anti-phosphotyrosine or an anti-paxillin antibody followed by rhodamine-conjugated secondary antibody, and F-actin was stained with fluorescein isothiocyanate-conjugated phalloidin. Cell–cell contact was examined by staining with an anti-E-cadherin antibody. IR produced double-strand break was detected by γ-H2AX antibody. Stained cells were then visualised with a confocal microscope. Scale bar, 15 μM. Data are representative of four or three (γ-H2AX) separate experiments.

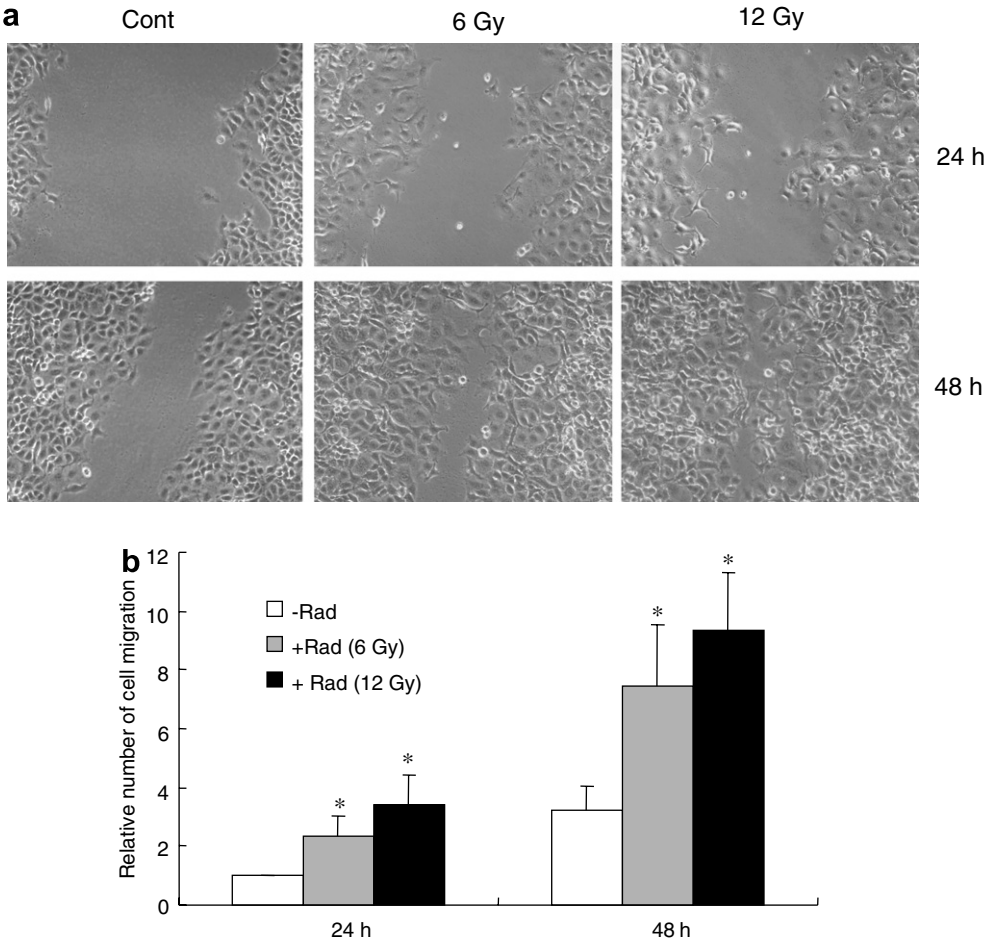

Fig. 3 – IR stimulates motility of A549 cells. (a) Cells were subjected to a single dose of IR (6 or 12 Gy) using a gamma cell irradiator, after which cell motility was measured with a wound healing assay. Changes in wound healing activity were visualised with a phase contrast microscope 24 or 48 h after irradiation. (b) A549 cells were subjected to IR (6 or 12 Gy), added to the upper compartments of a Boyden transwell chamber, and allowed to migrate. After 24 or 48 h, cells that migrated to the underside of the filters were counted. The fold increases (relative to control) of migrating cells are shown as the means of the relative cell numbers in ten randomly selected fields from a single representative experiment. The results in both panels A and B are representative of three independent experiments. The *error bars* indicate the SD $^{*}p < 0.05$ versus nonirradiated control cells.

3.4. *α-SMA, paxillin and TGF-β1 expression and FAK phosphorylation are up-regulated by IR*

Next, we measured the effect of IR on the expression of mRNA of α-SMA and vimentin, the hallmarks of EMT transition.[31,32] We observed that IR increased α-SMA mRNA expression, whereas changes in vimentin expression were not observed (Fig. 4a). Western blotting results demonstrated no significant increase of alpha-SMA protein level by IR up to 48 h (data not shown). That result indicates that IR may trigger some signals for SMA gene induction but not sufficient to express detectible amount of SMA proteins.

The production of TGF-β1, a main regulator of EMT, was then measured by ELISA before and after IR treatment. Induction of TGF-β1 was detected in culture medium 24 h after IR (Fig. 4b). In addition, the response of the A549 cells to TGF-β1 was assessed using a reporter assay using p3TP-lux reporter,[24] which is a chimeric reporter containing upstream regions from both the human plasminogen activator inhibitor 1 (PAI-1) gene[33] and the human collagenase gene.[34] IR increased the signalling of the cells to TGF-β (Fig. 4c). Exogenous TGF-β1 (5 ng/ml) had no detectible effect on cell morphology or migration (data not shown). Furthermore, TGF-β1 in combination with IR did not further enhance IR-induced morphology and migration changes (data not shown).

Two focal adhesion proteins, the focal adhesion-associated tyrosine kinase, FAK, and the scaffolding protein, paxillin, are critical for cell spreading and migration.[27] We found that IR increased paxillin expression and tyrosine phosphorylation of FAK (Fig. 4d).

3.5. *IR activates the p38 MAP kinase pathway in A549 cells*

Several signalling pathways may contribute to the regulation of EMT and the resulting change in motility. Therefore, we

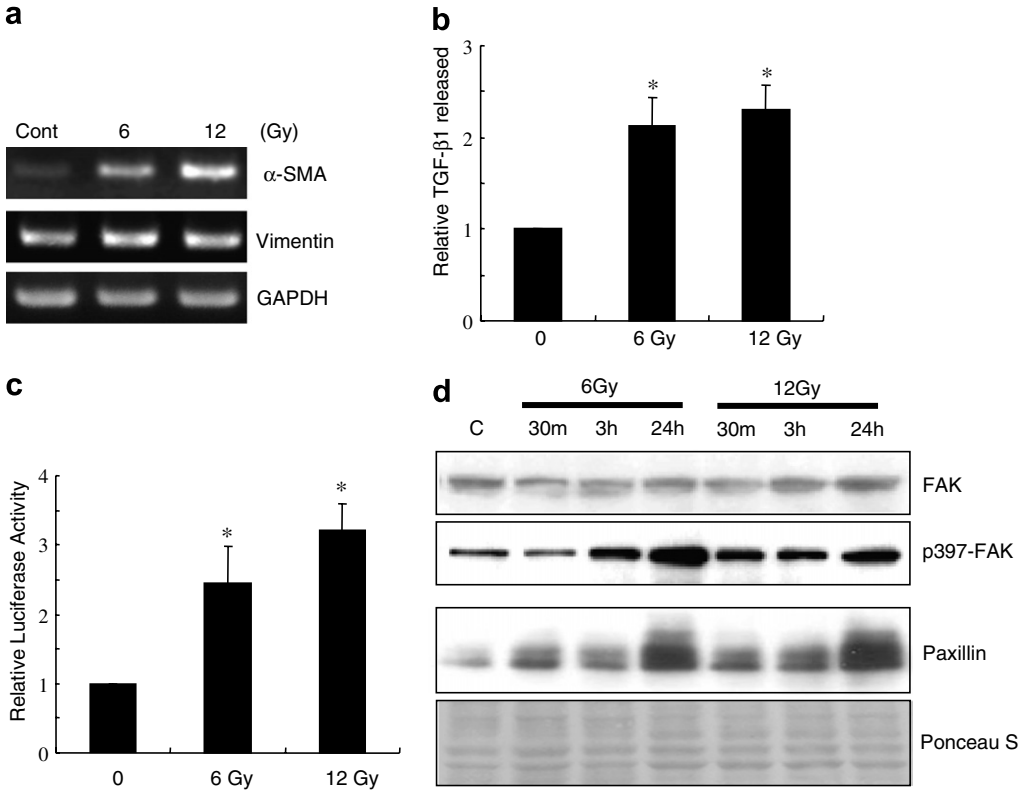

Fig. 4 – IR stimulates the accumulation of α-SMA mRNA, promotes the release of TGF-β, induces the protein expression of paxillin and FAK phosphorylation. (a) A549 cells were subjected to a single dose of IR (6 or 12 Gy) using a gamma cell irradiator. Total RNA was obtained 24 h later, and mRNA levels were measured by RT-PCR using specific primers. (b) Cells were subjected to a single dose of IR (6 or 12 Gy). After 24 h, the medium was collected, and the TGF-β1 level was determined by an enzyme immunoassay and the fold increases of TGF-β1 release are shown. The *error bars* indicate the SD of triplicate of two separate experiments $^{*}p < 0.05$ versus nonirradiated control cells. (c) A549 cells were transfected with p3TP-lux and CMV-βGal constructs and subjected to a single dose of IR (6 or 12 Gy). After 24 h, luciferase activity was measured. Also, β-galactosidase activity was measured to normalise for the transfection efficiency. The values are expressed as -fold increase over untreated cells. The *error bars* indicate the SD ($n = 3$) $^{*}p < 0.05$ versus nonirradiated control cells. (d) Cells were subjected to IR (6 or 12 Gy), and total cell lysates were obtained at various time points. Western blotting was performed using antibodies specific to FAK, phospho-FAK, or paxillin. Protein loading was verified by Ponceau staining.

next examined the activation of various MAPKs pathway by IR. Protein extracts were prepared at various time points from A549 cells before and after IR. Phosphorylation of p38 MAPK was detected after 30 min of IR treatment and reached a maximum at 3–6 h, whereas significant changes were not observed in the phosphorylation of ERK 1/2 or JNK MAPKs (Fig. 5).

3.6. *p38 MAPK is involved in IR-stimulated cell motility*

We next examined the role of the various MAPK pathways by testing the effect of specific inhibitors on cell migration during a wound closure assay and a transwell migration assay. Microscopic examination showed that cell elongation induced by IR in A549 cells was blocked by treatment with 10 μM SB203580 or SP600125, p38 MAPK- and JNK-specific inhibitors, respectively, but not by PD98059, an inhibitor of MEK1 (data not shown). Similarly, the p38 MAPK inhibitor prevented IR-enhanced cell motility (Figs. 6a and b). Also, SP600125 significantly suppressed migration both before and after IR. However, SB203580 inhibited IR-induced cell migration but not inherent cell migration. Finally, PD98059 had no detectable effect on cell migration.

4. Discussion

Although radiotherapy is a major therapeutic modality for cancer treatment, previous findings have suggested that IR promotes tumour migration, distant metastasis, and the invasive potential of cancer cells.[35–37] The results of the present study show that IR promotes cell migration and a series of changes that are associated with EMT in A549 human lung cancer cells. Importantly, enhancement of cell motility by IR was observed at doses clinically used for lung cancer. Recently, similar findings were reported by other investigators. Camphausen et al. reported that radiation therapy accelerated the metastatic growth of a primary Lewis lung carcinoma in mice.[37] Furthermore, Wild-Bode et al.[35] showed

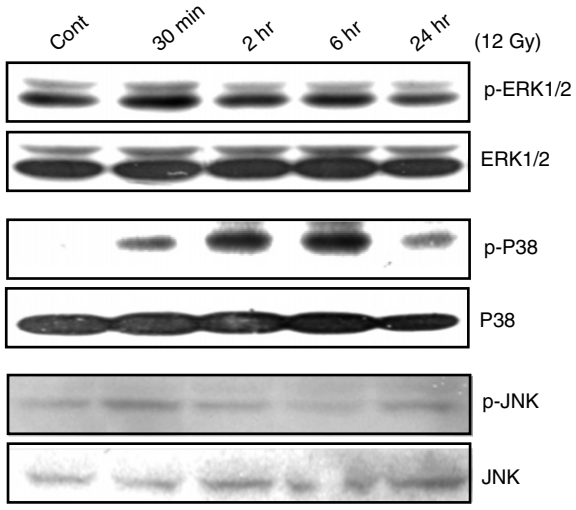

Fig. 5 – p38 MAPK is activated by IR. A549 cells were subjected to a single dose of IR (12 Gy), and total cell lysates were prepared at the indicated time points. Proteins (20 μg for ERK1/2, pERK1/2, p38, and p-p38; 60 μg for JNK and p-JNK) were separated by SDS-PAGE and analysed by immunoblotting with specific antibodies.

that sublethal doses of irradiation enhanced the migration and invasiveness of human glioblastoma cells in association with enhanced expression and activity of EMT-related molecules. These findings support our present results showing that radiation-mediated EMT is associated with enhanced migration and invasiveness. On the other hand, irradiation can inhibit cell proliferation and migration.[36] In fact, IR does not enhance cell migration or cause EMT-associated changes in H460 human lung cancer cells (our unpublished observations). Additional investigations are needed to elucidate the relevant molecular mechanisms behind the different responses to irradiation.

In our results, IR caused enhanced cell adhesion, actin cytoskeleton organisation and increased motility. These changes are all associated with cell transition from epithelial to fibroblast-like phenotype, characterised by enhanced cell motility. Fibroblast cells are not only more motile but also adhesive to ECM than epithelial cells. FAK and paxillin are two well-known proteins in focal adhesion, which ultimately regulate cell migration. IR-induced adhesion changes must be somewhat linked to increased paxillin expression and/or FAK phosphorylation. FAK phosphorylation at tyrosine 397 results in its direct interaction with Src, which is a critical mediator of integrin adhesion turnover to promote cell migration (for review[38,39]) and activates Src. We have discovered that Src activity was increased by IR in a dose dependent manner (data not shown). Further analysis to examine the role of FAK and paxillin in IR-induced EMT-associated or motility changes is certainly warranted.

Because EMT events require the coordinate expression of several sets of genes and signalling molecules, it is of importance to identify the molecular targets of signalling pathways that mediate the transcriptional regulation of these genes. Among these, TGF-β-associated signalling pathways are known to be key regulators of EMT. In this respect, it is noteworthy that radiation is a strong inducer of TGF-β both *in vivo* and *in vitro*.[40] The induction of EMT by TGF-β in A549 cells has also been reported.[41] Nonetheless, TGF-β by itself or in combination with radiation influenced neither radiation-induced cell motility nor EMT-associated changes (data not shown). Thus, the role of TGF-β in radiation-induced cell adhesion or motility changes is not evident in this study, probably in part due to the difficulty in reproducing the TGF-β response in cell culture. The cell status or culture conditions are probably very important parameters in the control of TGF-β function. It would be valuable to examine the influences of TGF-β suppression by neutralising antibody or siRNA or inhibition of TGF-β downstream signals on IR-induced changes in our future studies.

During motility, cells are in dynamic contact with extracellular matrix through integrins.[42] Therefore, IR-induced adhesion or motility changes in our system may be mediated by changed expression of integrins. It has been previously shown that IR strongly induced the expression of functional beta1-integrin and ILK in the two lung cancer cell lines, A549 and SKMES1.[43] Currently, however, how integrins are involved in radiation-induced adhesive phenotype or motility change is not known and remains to be investigated.

It has been shown previously that the activation of p38 by extracellular stress is mediated by reactive oxygen species[44,45] and IR may stimulate p38 by generation of ROS in A549 cells. Simultaneous administration of a p38 or JNK inhibitor blocked the radiation-induced increase in cell motility. These results are consistent with the proposed role of p38 MAPK in TGF-β-mediated cell migration and EMT of mammary epithelial cells.[46] P38 MAPK pathway is also shown to be a prerequisite for the activation of cell motility and the required cytoskeleton rearrangements.[15] It has been shown that exposure of cells to heat shock, strong oxidants, UV irradiation, IR irradiation, and other stressful conditions directly activate a family of stress-activated protein kinases, including p38 MAPK.[47,48] What aspect of EMT is mediated by p38 MAPK? It is suggested that p38 MAPK regulates actin organisation *via* HSP27.[49,50] In addition, it is possible that p38 MAPK contributes to the expression of TGF-β target genes that are involved in EMT because it has been implicated in TGF-β-transcriptional responses *via* activation of ATF2 and Sp1.[51,52] Although the role of p38 in radiation-induced EMT-associated changes remains to be elucidated, the results of this study suggest that the p38 MAPK signalling pathway plays a critical role in motility and presumably other EMT associated changes of A549 cells. A lot of evidence implicates that the JNK pathway is also important in regulation of cell migration in a broad range of cell types.[15,53–55] In order to find out the involvement of JNK activation for IR-induced cell migration, JNK activation was repeatedly assayed either by Western blotting using phospho-specific antibody or by *in vitro* JNK kinase assay, but failed to show its activation with appropriate positive control. Although JNK clearly regulated cell motility of A549 cell based on inhibitor experiment, the activity regulation JNK by IR is not obvious. JNK may be constitutively activated and regulate both inherent and IR-induced cell migration.

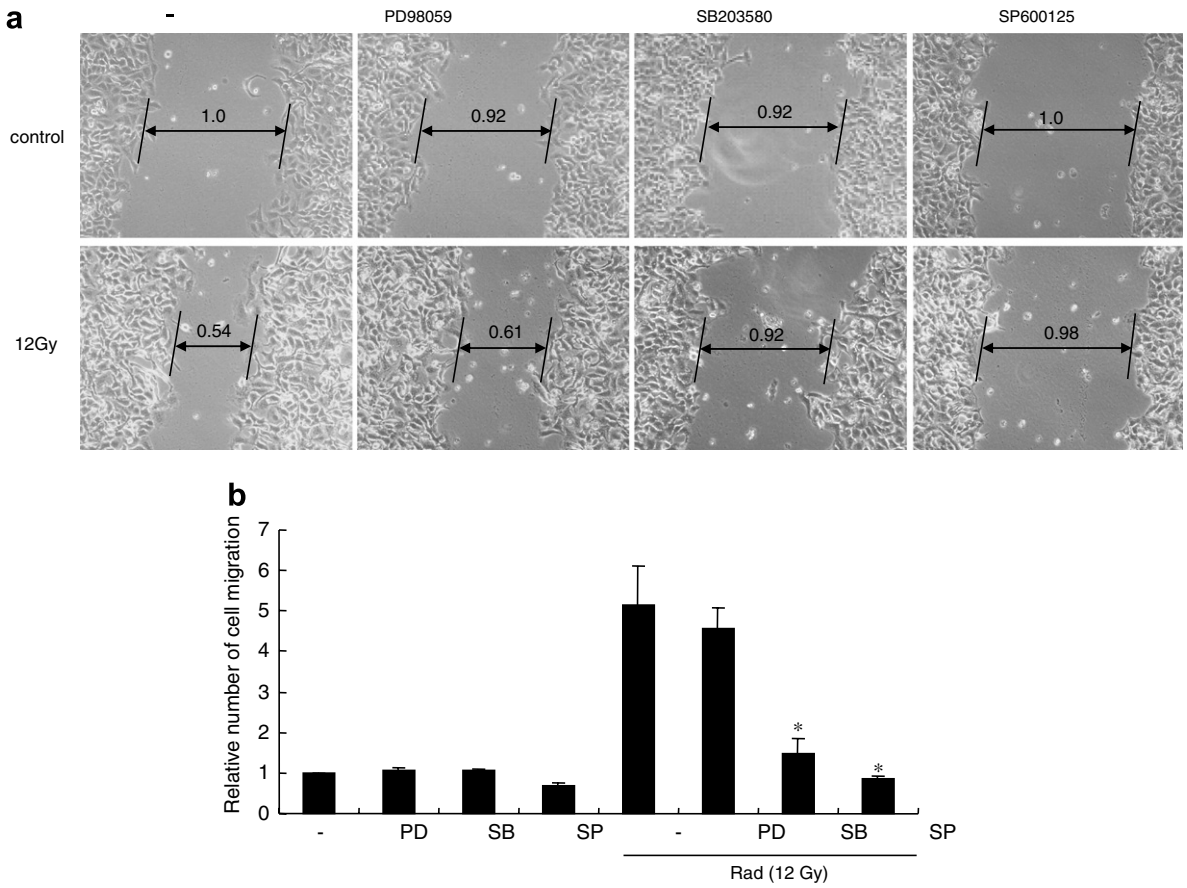

Fig. 6 – p38 MAPK participates in IR-mediated cell migration. (a) A549 cells pretreated with PD98059 (10 μM), SB203580 (10 μM), or SP600125 (10 μM) for 30 min before being subjected to a single dose of IR (12 Gy). Changes in wound healing activity were visualised with a phase-contrast microscope 24 h after the injury. The relative distance of wounding area was depicted. (b) A549 cells (1×10^5/well) were seeded in the upper chamber of 5-μm pore transwells, and inhibitors (10 μM of PD98059, SB203580, or SP600125) were added 30 min before exposure of the cells to IR (12 Gy). Cells that invaded the underside of the filters were counted after 24 h. The fold increase (relative to control) of migrating cells is shown based on the mean of relative cell number in ten randomly selected fields from a single representative experiment. The results in both panels A and B are representative of three independent experiments. The *error bars* indicate the SD of fold decrease $^*p < 0.05$ versus irradiated cells.

The fundamental question of the importance of EMT in radiation fibrosis remains. In other words, is EMT essential for radiation fibrosis in normal or cancer cells? The answer is not clear at this point. Nonetheless, the reorganisation of the actin cytoskeleton, induction of α-SMA, and acquisition of a transformed morphology during the process of EMT may provide a structural foundation for fibrosis. In fact, a series of IR responses leading to radiation fibrosis or radiation metastasis are result of chronic long-term changes and a lot of additional factors released by neighbouring cells may be required to induce more dramatic and intact EMT-associated changes *in vivo*. Nonetheless, this study has some value in establishing cell study system to investigate the mechanism of acute EMT-associated cellular changes to therapeutic radiation.

Conflict of interest statement

None declared.

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